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Commentary

Flower development and evolution: New answers and new questions

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Certain genes have a way of rewarding continued study, as can be seen from the long histories of discoveries that have resulted from work on mammalian hemoglobin genes, on the *Escherichia coli lacZ* gene, and on many others. A plant gene that may fit this mold is the homeotic flower-development gene AGAMOUS (*AG*). In the past few years, study of the genetics and molecular genetics of this *Arabidopsis thaliana* gene and of its orthologues in other plant species has led to a stream of discoveries that have revealed some of the mechanisms and some of the complexity of flower development. Two recent papers (1, 2), one in this issue, describe a new level of regulation of *AG* and raise new questions about the function and evolutionary history of this gene.

Action and Expression of AGAMOUS

The phenotype of agamous (*ag*) mutants has been familiar to gardeners for millennia, since loss of function of the gene leads to double flowers (flowers with more than the usual number of petals), which have been grown as ornamentals since ancient times (3). The wild-type pattern of floral organs in an *Arabidopsis* flower is four sepals surrounding four petals, which themselves surround six stamens and a central pistil. *ag* homozygotes have the six stamens replaced by six petals and the pistil replaced by an inner flower with the same structure as the outer (4–6). The flower consequently has, from outside to center, the repeating pattern (four sepals, four petals, six petals)$_n$. Therefore, *AG* is responsible for two important aspects of floral development: it is required for the normal development of stamens and carpels (the subunits of pistils) and for determinacy (cessation of organ proliferation, leaving no remnant of the generative meristem). Both carpels and determinacy are defining characteristics of flowers. Because of the apparent conversion of stamens to petals and of carpels to either new sepals or new flowers (depending on how one wishes to interpret the indeterminacy), *AG* is classed as a homeotic, or organ identity, gene (4–7). Genetic studies have shown that *AG* has in fact three functions, which are separately revealed in different genetic backgrounds: provision of determinacy, contribution toward stamen and carpel identity, and negative regulation of the activity of a set of genes that contribute to petal and sepal identity (7–9).

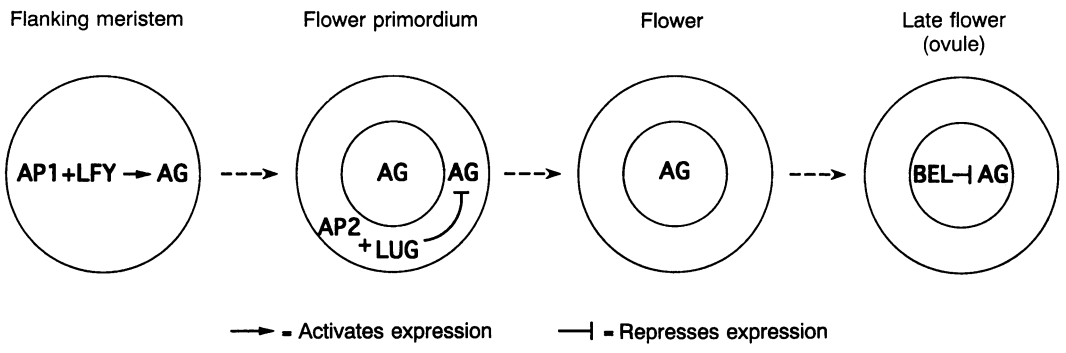
The molecular cloning of *AG* was reported in 1990 (10). Its product is a member of what has come to be called the family of MADS domain proteins because of a conserved DNA-binding motif that was initially described in products of the *Saccharomyces* gene *MCM1*, the *Arabidopsis* gene *AG*, and the snapdragon gene DEFICIENS (*DEF*) and in the human protein serum response factor (11). The AG protein binds to DNA in a sequence-specific manner, recognizing the double-stranded sequence represented by 5′-TTDCCW$_6$GGNAA-3′ in which D = A, T, or G, W = A or T, and N = A, T, C, or G (12, 13). *In situ* hybridization to wild-type flowers shows that the gene is expressed in a pattern that changes as floral development proceeds (14, 15). The first appearance of the RNA is at an early stage of flower development, prior to the stage when the primordia of petals, stamens, or carpels appear. At this stage, AG RNA is found in the central region (whorls 3 and 4) of the floral primordium, where stamens and pistil will later form. As flower development continues, the AG RNA remains present in developing stamens and pistil until the differentiation of microsporocytes in the anthers, and of ovule primordia in the developing ovary. The RNA then selectively disappears from these regions, becoming absent in forming pollen grains and in developing ovules, except for one specialized cell type in the ovules, the endothelial cells. The RNA is finally reduced in all developing organs, remaining highly expressed only in small regions of the anther, in endothelial cells, and in the stigma cells that cap the ovary and style. The early expression pattern clearly relates to the mutant phenotype: the RNA appears just before the mutant phenotype does, and in the same domain. The later function of AG, if any, is not known, because eliminating the activity of the gene normally eliminates the cell types in which later expression is found.

The ectopic expression of *AG* in *Arabidopsis* demonstrates the function of the gene that is inferred from its mutant phenotype (16). If *AG* is expressed from a viral promoter so that it is active in all cells of the developing flower, one finds carpels where sepals would usually be and staminoid organs in place of petals. This is because AG represses the action of the genes that normally contribute to sepal and petal identity and at the same time promotes stamen and carpel identity.

Continued genetic and molecular study of the expression pattern of *AG* has revealed a complex set of positive and negative regulators, which assure that stamens and carpels develop at appropriate times and in appropriate floral regions (Scheme I). The initial induction of *AG* depends on two positive regulators, which act redundantly on *AG* (17). These are the meristem identity genes LEAFY (*LFY*) and APETALA1 (*AP1*), which come on earlier in flower development than *AG*, in a domain that includes the cells that will give rise to all floral organs. The question of how *AG* manages to come on only in the central region of the flower when its inducers are active all over the flower has been partially answered by the discovery of a pair of spatially-specific negative regulators of *AG* (ref. 14; Z. Liu and E.M.M., unpublished data). These negatively acting genes (cadastral genes in the *Arabidopsis* terminology) are APETALA2 (*AP2*) and LEUNIG (*LUG*). Mutations in either result in AG RNA accumulating everywhere in the developing flower. The phenotype of this is the same as that of ectopic *AG* expression: carpels replacing sepals and stamens replacing petals (refs. 6–8, 18; Z. Liu and E.M.M., unpublished data). These genes (*AP2*, at least) are not responsible for the specific disappearance of AG RNA from developing floral cell types late in flower development: in *ap2* mutants stamens and carpels show normal patterns of late *AG* expression (15).

Late Expression Is Specifically Regulated

The work reported in this issue of the *Proceedings* (1) and in a recent paper in *The Plant Cell* (2) demonstrates the importance of the late disappearance of *AG* from developing ovules, reveals a new *AG* regulatory gene, and brings to the fore evolutionary questions about the function of *AG* and its relation to the



Scheme I

origin of flowers. Ovules develop in stages, with a multicellular projection, the nucellus (later to be the site of female meiosis) forming in the ovary, followed by the successive development of two integuments from the periphery of the nucellus. The integuments later form the seed coat. The outer integument forms the outer coat of the seed, and the inner integument is apposed to the nucellus. The inner cell layer of the inner integument is the *AG*-expressing endothelial layer, as mentioned above. Robinson-Beers *et al.* (19) earlier described a gene, BELL (*BEL*), whose mutations cause abnormal integument formation. Instead of the wild-type two integuments, *bel* mutants initiate only a single integument (that in its inception resembles the outer integument). This then develops to an abnormal structure. Ray *et al.* (1) and Modrusan *et al.* (2) continue this work by finding strong mutant alleles of *BEL*, presumably representing a more complete loss of function than the earlier alleles. In these strong *bel* mutants, it is clear that the single structure that forms in place of the integuments is a carpel, with characteristic carpel cell types, including stigmatic cells at its apex. Furthermore, the embryo sac, which normally forms in the nucellus after meiosis, is absent.

The ectopic appearance of carpel tissue suggests that abnormal ovule development in *bel* mutants may be due to ectopic expression of *AG*. *In situ* hybridization confirms that in *bel* mutants, AG RNA is present at high levels throughout developing ovules. To further establish causal relationships, Ray *et al.* caused *AG* to be overexpressed from a viral promoter as in earlier experiments, but they carefully observed the fate of the ovules in the transgenic plants. The ovules developed as in *bel* mutants. This work thus reveals the first known regulator of the late pattern of *AG* expression: *BEL* is a negative regulator of AG RNA accumulation that acts in ovules at late stages of flower development to eliminate AG RNA from all cells except the endothelial cells. Without this action, the ovules will develop to carpelloid fates.

Like the *AG* mutant phenotype, this type of teratological ovule phenotype has been recognized in many species and for many years. One heritable example in *Primula* was described in detail by de Candolle and de Candolle in 1841 (20); they also described instances of carpelloid development of ovules in *Cheiranthus*, which is, like *Arabidopsis*, a member of the mustard family. Another instance was described in carnations in 1850, where the ovules (with a single covering, as in *bel* mutants) are converted to carpels, and these carpels themselves bear ovules. This abnormality is described in Masters' 1869 treatise on plant teratology (21), with the comment "Could such a change occur in the animal kingdom, there would be the unfertilised ovum converted into an ovary, and this again bearing Graafian vesicles!"

Evolutionary Issues

Two evolutionary questions are raised by the study of *AG* and by the action of *BEL*. One is the question of whether stamen, carpel, and ovule developments are regulated by an orthologue of *AG* in species other than *Arabidopsis*. The answer is yes. Genes with sequences closely related to that of *AG* have been cloned from *Brassica napus*, a mustard related to *Arabidopsis*; *Antirrhinum majus* (snapdragon); *Nicotiana tabacum* (tobacco); *Petunia hybrida* (garden petunia); and *Lycopersicon esculentum* (tomato) (22–27). In all of these cases, the early expression pattern of the gene is very similar to that of *AG* in *Arabidopsis*. In snapdragon (23), the gene has been shown to correspond to the mutation *plena* (*ple*), the phenotype of which is very similar to that of *ag* in *Arabidopsis*. Furthermore, a transposon-induced gain-of-function mutation at this gene causes the carpelloid sepals and staminoid petals caused by *AG* constitutive expression in *Arabidopsis*. Transformation experiments with sense and antisense orientations of the *AG* homologue have indicated a comparable function for the cloned homologue in tobacco, petunia, and tomato (24, 26, 27). In fact, experiments in which the *Brassica AG* gene was introduced to tobacco with a constitutive promoter show that the gene from one species causes the expected phenotype in a distantly related species, which underlines the orthology of the genes (22). In addition, in these *Brassica*/tobacco experiments, overexpression of *AG* was shown to create a tobacco ovule phenotype like that of *bel* mutants in *Arabidopsis*. This shows that the late as well as the early function of the gene is conserved. All of the plants in which *AG* homologues have been shown to serve the *AG* function are, so far, advanced dicots. *AG* relatives have also been found in the more distantly related monocot *Zea mays* (maize; ref. 28). In this case two genes have been identified that are close relatives of *Arabidopsis AG*. The expression pattern of one of them is very close to that of *AG* in *Arabidopsis*, indicating that this may be the maize homologue of *AG*.

Another evolutionary question raised is that of the ancestral function of *AG*, and its possible role in the origin of flowers. Among the functions of *AG* and its orthologues are the common establishment of stamens and carpels and the imposition of determinacy. These (particularly bisexuality and carpels) are characteristics of flowers, and not of more primitive plant reproductive structures. The acquisition of the present functions of *AG* would thus appear to be key steps in the evolution of flowers (whose origin is surprisingly recent: flowers first appear in the fossil record in the Lower Cretaceous, around 120 million years ago). What is the origin of *AG* function? That it is a member of a gene family found in three kingdoms indicates an ancient origin for the class of proteins that includes AG; that orthologous genes are found in many species of flowering plants indicates that *AG* was present in their ancestors. Is there a similar gene, with related functions, in nonflowering plants? Or is the ancestral gene one with a different function, adopted for altogether new functions when flowers originated? Could the primitive function be more closely related to *bel* and ovule

development, with the developmentally early functions being evolutionarily late? If so, we might begin to explain the existence of the BELL–AGAMOUS regulatory interaction. If *AG* had some ancestral function in ovules, as that function changed to include the specification of carpels, it might have become advantageous to repress *AG* in developing ovules. But what, then, was the ancient function of the *BEL* ancestor?

We know too little to answer any of these questions, although experiments to find *AG* relatives in nonflowering and primitive flowering plants are in progress (refs. 29 and 30; M. Frohlich and E.M.M., unpublished data). We also know little about the molecular details of *AG* regulation. It is induced by *LFY* and *AP1*, repressed in the periphery of early developing flowers by *AP2* and *LUG*, and repressed in developing ovules by *BEL*. Do these regulators act directly or through as-yet-unknown intermediates? Where in the *AG* gene are the enhancers through which these genes act? And what are the genes to whose promoters the AG protein binds, so that *AG* can carry out its functions in organ specification and determinacy? That these questions are still open shows that there are many more answers yet to come from continued study of AGAMOUS.

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